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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/68, C07K 1/04 // G01N 33/86	A1	(11) International Publication Number: **WO 97/42507** (43) International Publication Date: 13 November 1997 (13.11.97)

<table>
<tr>
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(21) International Application Number: PCT/GB97/01228

(22) International Filing Date: 2 May 1997 (02.05.97)

(30) Priority Data:
9609262.2   2 May 1996 (02.05.96)   GB

(71) Applicant (for all designated States except US): ISIS INNO-VATION LIMITED [GB/GB]; 2 South Parks Road, Oxford OX1 3UB (GB).

(72) Inventors; and
(75) Inventors/Applicants (for US only): ESNOUF, Michael, Peter [GB/GB]; Selbourne, 7 Stonesfield Road, Combe, Witney OX8 8PF (GB). GAO, Bin [CN/GB]; 12 York Avenue, Headington, Oxford OX3 8NS (GB).

(74) Agent: PENNANT, Pyers; Stevens Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).

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(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

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(54) Title: PEPTIDE ARRAY AND METHOD

(57) Abstract

A method of studying a functional protein comprises providing an array of support-bound oligopeptides with different oligopeptides bound in an immunoreactive state at different locations on the support wherein each oligopeptide has defined amino acid residues at two chosen positions and a mixture of amino acid residues at other positions; applying the functional protein in solution to the array; and identifying support-bound oligopeptides that bind to the functional protein. Secondary oligopeptide libraries are used to further elucidate the amino acid sequence of the target protein. The method is used to elucidate the core residues responsible for the binding of a monoclonal antibody to its antigen β-factor XIIa.

PEPTIDE ARRAY AND METHOD

5   Elucidation of the interactive residues of a functional protein
with its peptide ligand provides important information for understanding
and manipulating the biological processes in which the functional protein is
involved. Combinatorial peptide libraries are powerful tools for identifying
these interactive sequences. Libraries comprising large numbers of
10   peptides ranging from 10^6 - 10^{12} are available for this purpose. But such
libraries are necessarily complex and expensive.

Combinatorial peptide libraries have also been built up on
beads by a "split-mix" approach, which ensures that each bead carries only
one sequence. A difficulty with this approach is that the identity of the
15   peptide sequence on a particular bead is not known and must be
determined by analysis. Such analysis is time consuming and may be at
the limits of available technology.

A "one step" positional scanning approach to identify a
peptide ligand has been reported (see R A Houghton et al, Biotechniques,
20   13(6), 901-5). In this technique, a library was made up with peptide
mixtures each with an amino acid defined at one position and the other
positions containing a mixture of 19 amino acids. H M Geysen describes in
US Patent 5,194,392 a corresponding technique in which each
oligopeptide of a library contains known amino acid residues at two
25   specified positions. When contacted with a functional protein in solution,
only those peptides with the correct amino acids in each position gives the
strongest recognition signal. However, individual oligopeptides of each
library were mounted on beads or on pins, which is disadvantageous since
it requires the use of a rather large amount of functional protein in solution.
30   Also, it is difficult to ensure that each oligopeptide of the library is

incubated with the functional protein under identical conditions.

This invention is concerned with a membrane-based approach in which different peptide ligands are bound at different locations on a support. This has the advantage that the identity of the peptide ligand at a particular location is at least partly known. A disadvantage is that, with 20 different natural L-amino acids to choose from, an array of all possible oligopeptides, even short ones, would be impossibly large and expensive to produce. A strategy is provided for limiting the size of an array.

Thus in one aspect the invention provides a method of studying a functional protein, which method comprises

a)   providing an array of support-bound oligopeptides, with different oligopeptides bound in an immunoreactive state at different locations on the support, wherein each oligopeptide has defined amino acid residues at two chosen positions,

b)   applying the functional protein in solution to the array and identifying a first support-bound oligopeptide that binds to the functional protein,

c)   using the information generated in b) to study the functional protein.

The invention also provides an array of support-bound oligopeptides, with different oligopeptides bound in an immunoreactive state at different locations on the support, wherein each oligopeptide has defined amino acid residues at two chosen positions.

Thus a library of oligopeptides is provided in an array on a support. Generally the support will be a membrane or a flat plate. The support needs to be generally hydrophilic, so as to ensure that each oligopeptide of the array is tethered in an immunoreactive state, and to be resistant to DMF and other solvents used in oligopeptide synthesis. The inventors have used a polyvinylidenedifluoride membrane with success but others are possible, for example polyamide such as Nylon 66 and even

paper. The support may be activated, either to provide carboxyl groups for reaction with amine groups of amino acids, or more preferably to provide amine groups for reaction with carboxyl groups of amino acids. For example, a support may be reacted with ethylene diamine to provide

5 primary amine groups. To reduce any problems of steric hindrance, a linker molecule may be provided between the support and the first amino acid residue of each oligopeptide. Thus for example an unnatural amino acid such as β-alanine may be provided on the surface for reaction with the first amino acid of each oligopeptide member of the array. Techniques for

10 synthesising oligopeptides on a support are well known and do not form part of this invention. Preferably the amino acid residues of each oligopeptide of the library, at all positions other than the two chosen positions, are mixtures of all natural L-amino acids except cysteine. Such arrays are readily generated by combinatorial chemical methods.

15   The support is preferably planar. Alternatively the individual locations of the support may be the individual wells of a microtitre plate. The array may comprise (19 x 19) 361 different oligopeptides bound at 361 different locations on the support. The oligopeptide at each different location would comprise a different combination of two defined natural L-

20 amino acid residues excluding cysteine which may be the same or different.

Although different oligopeptides of the array may have different lengths, it will usually be convenient for all oligopeptides of the array to be the same length. This should be great enough to permit

25 effective binding to an epitope of the functional protein, that is to say generally comprising at least five or six amino acid residues. Longer oligopeptides may lose flexibility or develop structure of their own which make them less able to bind to epitopes of the target functional protein. Preferred length is 5 to 12, e.g. 7 to 9, amino acid residues.

30   The positions of the two known amino acid residues in these

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oligopeptide members of the array can be varied, generally the known
residues will occupy the same positions in each oligopeptide member of
the array. Preferably these are non-adjacent positions. For example,
where each oligopeptide is support-bound through its C-terminus, the
5   defined amino acid residues may be at the 2- and 4-positions counted from
the C-terminus.

The target functional protein may be an antibody, or may be
any other protein which binds specifically by an immune or non-immune
reaction to another peptide sequence. For example the target may be an
10   antigen, and the oligopeptides of the array may represent its associated
antibody. In use, the target functional protein in solution is applied to the
array of support-bound oligopeptides, under conditions which permit the
target to bind to one or more oligopeptides in the array.

The method of the invention involves identifying at least one
15   support-bound oligopeptide that binds to the functional protein in this way.
This may readily be achieved by labelling the functional protein, either after
or before applying it to the array of support-bound oligopeptides. We have
used an anti-Ig-G-peroxidase conjugate as label, applied after binding an
antibody target to the array, followed by an enhanced chemiluminescent
20   detection system with success, though a chemiluminescent alkaline
phosphatase system is an alternative and other detection systems are
possible. Or the target in solution may be labelled with any radioactive,
chromogenic or fluorescent signal moiety or with a component of an
enzymatic signal-generating system. By means of the label, the location of
25   the target protein on the array, and hence the identity of the oligopeptide to
which it has bound, are easily determined.

That oligopeptide contains two known amino acid residues at
known positions, which may correspond to amino acid residues in a
biological receptor for the functional protein. This information is then used
30   to further study the functional protein, for which several strategies are

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possible. According to a preferred strategy, this step is performed by constructing several secondary libraries of support-bound oligopeptides in which: each support-bound oligopeptide of each secondary library has the same amino acid residues at the same two chosen positions as the first

5   support-bound oligopeptide; each support-bound oligopeptide of a secondary library has a defined amino acid residue at another chosen position; and the said other chosen position is different for each secondary library.

The target functional protein in solution is applied to each of

10   these secondary libraries. This permits identification of a secondary support-bound oligopeptide in each secondary library that binds to the functional protein. Each secondary support-bound oligopeptide carries a known amino acid residue at a known position. From the information collected from all these secondary library experiments, it is possible to

15   build up a substantially complete picture of an immuno dominant epitope of a (hypothetical) receptor that binds to the target functional protein.

Alternatively, other strategies are possible at this secondary stage of the method. For instance, secondary libraries of support-bound oligopeptides can be prepared in series, rather than in parallel, with the

20   oligopeptides of each library containing one or more known amino acid residue at a known position than the oligopeptides of the previous library. Alternatively a secondary library of oligopeptides on beads can be prepared by a "split-mix approach", provided that the oligopeptide on each bead carries the two defined amino acid residues at the two positions

25   chosen in the first stage.

Preferred membranes based on polyvinylidenedifluoride have several advantages. They are easily derivatised to enable oligopeptides to be stably bound through the N-terminus, in a form which is immunoreactive with a target functional protein in solution. When used in this way, the

30   membrane is found to have very low background noise, so that the

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signal-to-noise ratio is large. Further, once an adsorbed target functional protein has been eluted, the membrane can readily be re-used; as described in the examples, a preferred membrane was re-used 20 times without noticeable deterioration.

Reference is directed to the accompanying drawings in which:-

Figure 1 is a bar chart showing the binding intensity of different peptide mixtures in an array for a monoclonal antibody.

Each of Figures 2a to 2f is a bar chart showing binding of the monoclonal antibody to a secondary library of oligopeptides.

Figure 3 is a diagram of a scanning array of overlapping oligopeptides based on the sequence of β-factor XIIa, showing regions which bind to various antibodies and inhibitors.

Example 1 below demonstrates the successful use of the above method to elucidate the core residues responsible for the binding of the monoclonal antibody 201/9 to its antigen β-factor XIIa.

EXAMPLE 1

Membrane preparation

A sheet 10 x 12 cm of Immobilon-AV-1 membrane (Millipore, UK was immersed in 20 ml of acetonitrile and 4 ml of ethylenediamine (Aldrich, UK) and 200 μ1 triethylamine (Aldrich, UK) and reacted at room temperature overnight (15 hours.). The membrane was washed three times for 5 mins with 20 ml of acetonitrile and then three times with 20 ml of methanol and dried with a stream of cold air. 18.6 mg of Fmoc-B-Ala (0.3 mM, Novabiochem, UK), 12 mg of N-hydroxybenzotriazole (0.45 mM, Fluka, UK) and 9 μl of N,N'-Diisopropylcarbodiimide (0.36 mM, Aldrich, UK) were dissolved in 200 μl 1-methyl-2-pyrrolidone (Fluka, UK) and incubated at room temperature for 30 mins to activate the amino acid and then 0.5 μl of the mixture was spotted at 4 mm intervals on the membrane. After

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20 mins when the coupling was complete, the membrane was washed
three times with 20 ml amine-free N,N-dimethylformamide (Fluka, UK) and
the unreacted amino groups were blocked by acetylation with 4%(v/v)
acetic anhydride in N,N-dimethylformamide for 10 minutes. The protecting
Fmoc group was then removed from the β-alanine with 20 ml of 20%(v/v)
piperidine (Fluka, UK) in N,N-dimethylformamide for 5 mins at room
temperature after which the membrane was washed 5 times with 20 ml
N,N-dimethylformamide. To monitor the efficiency amino-acid coupling the
membrane was stained with 0.01%(w/v) bromophenol blue solution in N,N-
dimethylformamide for 5 minutes before the addition of the activated
amino-acid. The stained membrane was washed three times with 20 ml
methanol and finally dried under a stream of cool air. During the formation
of the peptide bond the colour of the spot changed from blue to yellow-
green. Free amine was removed from the solvents used for peptide bond
formation by storing them over molecular sieve type 4A.

Construction of the peptide libraries.

The libraries were constructed on two sheets of derivatised
membrane. On the first membrane (1 x 12 cm) a 19 x 19 matrix of peptide
mixtures was constructed making a total of 361 groups of peptides.
Positions 2 and 4 of the peptide sequence contained defined amino acids,
while at each of the remaining positions was added a mixture of the 19
natural amino acids (except cysteine) in equimolar proportions. Having
established the amino acids in positions 2 and 4 which gave the best
antibody binding in the first scan these were then used in a second scan.
The peptides in this scan were synthesised using the so-called "positional
scanning approach", but incorporating in positions 2 and 4 the amino acids
identified in the first scan. In the second scan one of the 19 natural amino
acids was incorporated into a third position while at the remaining 5
positions in the octapeptide was added a mixture of all the amino acids.

The libraries can be represented as O_1-D-X-D-X-X-X-X;

X-D-O_2-D-X-X-X-X; X-D-X-D-O_5-X-X-X; X-D-X-D-X-O_6-X-X;

X-D-X-D-X-X-O_7-X and X-D-X-D-X-X-X-O_8. O represents one of 19 amino

acids, X represents the mixture of 19 amino acids and D represents the

5   amino acids identified from a screen of the first set of libraries. The

peptides were synthesised using a modified coupling cycle as follows: (I)

Amino acid coupling:- Each of the Fmoc-amino acids (Novabiochem, UK)

to be coupled was dissolved in 200 µl of 1-methyl-2-pyrrolidone containing

0.45 mM N-hydroxybenzotriazole and 0.36 mM N,N'-

10   Diisopropylcarbodiimide and the final concentration of the activated amino

acid was 0.3 mM and 0.5 µl of each activated amino acid, or amino acid

mixture, was added with a micro-pipette to each spot on the membrane.

After 30 minutes at room temperature, the membrane was washed three

times with 20 ml of N,N-dimethylformamide. (II) Acetylation of the N-

15   terminus:- After each coupling cycle any free amino groups were

acetylated by soaking the membrane in 4%(v/v) acetic anhydride in N,N-

dimethylformamide for 15 mins and the excess acetic anhydride decanted.

The membrane was washed with N,N-dimethylformamide (20 ml x 3),

followed by dichloromethane (20 ml x 3, BDH, UK). (III) Fmoc

20   deprotection:- The membrane was agitated for 5 minutes with 20 ml of

20% (w/v) piperidine in N,N-dimethylformamide after which the piperidine

was removed and the membrane was washed with N,N-dimethylformamide

(20 ml x 6) and any remaining amino groups were acetylated as described

above. After the final coupling step the Fmoc protection was removed and

25   the free amino groups were acetylated. To deprotect the side chains the

membrane was incubated in 20 ml of 50% (v/v) trifuoroacetic acid (Fluka,

UK and 2.5% (v/v) triisobutylsilane (Aldrich, UK) in (v/v) trifluoroacetic acid

(Fluka, UK and 2.5%(v/v) triisobutylsilane (Aldrich, UK) in dichloromethane.

After 1 hour at room temperature the trifluoroacetic acid solution was

30   decanted and the membrane was washed three times with 20 ml

dichloromethane and then with N,N-dimethylformamide (20 ml x 3)
followed by methanol (20 ml x 3) and after drying was stored at 4°C.

Immunoblotting for locating recognition residues.

5   Before probing the peptides with the antibody, the membrane
was wetted with 20 ml methanol and transferred into 20 ml 0.01 M
phosphate buffered saline pH 7.4 with 0.02% (w/v) Tween-20 for three
minutes. The membrane was blocked in 20 ml 10% (v/v) new-born calf
serum in 0.01 M phosphate buffered saline with 0.02% (w/v) Tween-20,

10   pH 7.4 for two hours. The membrane was washed with (3 x 20 ml) 0.01 M
phosphate buffered saline pH 7.4 containing 0.02% (w/v) Tween-20. The
appropriate concentration of ascites fluids or purified IgG, containing the
murine monoclonal antibody 201/9 against human β-factor XIIa was
incubated with the membrane for 2 hour at room temperature. The

15   membrane was washed and finally incubated with an optimised anti-mouse
IgG-peroxidase conjugate in 20 ml of 0.01 M phosphate buffered saline pH
7.4 containing 0.02% (w/v) Tween-20 for one hour at room temperature.
After extensive washing with 0.01 M phosphate buffered saline with
0.02% (w/v) Tween-20, pH 7.4 and then buffer (3 x 20 ml) without Tween-

20   20, the membrane was subjected to signal development. The membrane
was first blotted on tissue paper and rinsed in an enhanced
chemiluminescent solution (0.05 M borate buffer pH 8.5 containing 0.4 mM
4-Iodophenol (Fluka, UK) and 1.25 mM Luminol (Sigma, UK) and 2.7 mM
H_2O_2 (Sigma, UK)) for 30 sec. The membrane was wrapped with cling film

25   (Lakeland Plastics, UK) and exposed to a light sensitive film, Hyperfilm
(Amersham, UK) in a light enhance cassette (CAWO, Germany) for
different times depending on signal intensity. The films were developed in
Develop-74 (Kodak UK) for 2.5 mins at room temperature and 5 minutes in
a fixer solution (Kodak UK). The film was extensively washed with water

30   and dried in the air. The film with the image of the spots was scanned with

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an ARCUS PLUS scanner (Agfa, UK) using the Photoshop program on a
Macintosh Quatra 950 computer and the intensity of the spots was read
with NIH Image.

5   **Re-use of the membrane.**

The bound proteins were stripped from the membrane with 20 ml of
1% (w/v) sodium dodecyl sulfate (BDH, UK) containing 8 M urea (BDH,
UK) and 100 mM mercaptoethanol (BDH, UK) for 30 minutes. The
membrane was then washed extensively with distilled water and finally with
10   0.01 M phosphate buffered saline, pH 7.4.

RESULTS

Construction of randomized libraries.

To identify the residues of β-factor XIIa, the protease domain
15   of activated coagulation factor XII, involved in the binding of the
monoclonal antibody 201/9, randomized peptide libraries were constructed
on a piece of polymer membrane. The peptide mixtures were synthesised
in a 19 x 19 matrix on each spot on a piece of polymer membrane. The
strategy for the synthesis of the peptides was as outlined above. In short,
20   residues number 2 and 4 were chosen as the defined amino acids in the
libraries, the sequence of each peptide can be represented by the by
formula $X-O_2-X-O_4-X-X-X-X$. The peptides were synthesised in each
column with position 4 (O_4) containing one of 19 the natural amino acids
(cysteine omitted) on each spot and in each row with position 2 (O_2)
25   defined as one of the 19 amino acids, while a mixture of 19 amino acids
was used at the other positions. The whole membrane contained
approximately 1.69×10^{10} peptides in total and in each spot there were
4.7×10^7 peptides. The membrane with the peptide libraries was incubated
with monoclonal antibody 201/9. The antibodies bound to the peptide
30   mixture were then reacted with an anti-mouse IgG-peroxidase conjugate

and the signal was amplified by an enhanced chemiluminescent assay. The results showed that the spots with the strongest recognition contain Phe and Gln or Ile and Ile in positions 2 and 4 respectively. The sequences of the peptides and the strength of recognition is presented as a grey scale (Fig. 1).

Since each recognised spot was composed of a calculated 4.7×10^7 (19^6) peptides and only the amino acid at positions 2 and 4 were defined, a further series of iterative experiments is required to identify all the residues involved in the binding of the antibody. For this, it would be necessary to incorporate each of the 19 amino acids at the six remaining six positions in the octapeptide sequence. To reduce the number of iterative syntheses a positional scanning technique was adopted. The two amino acids, identified in the first screening library, were incorporated together with a defined amino acid in a third position into the new libraries. The two best recognised peptide mixtures (Fig.1) were chosen for further analysis. These contained in the defined positions 2 and 4 either Phe and Gln in the first peptide or Ile at both positions in the second. To identify the remaining residues of the epitope 12 positional peptide libraries, differing only in the location of the defined position, were synthesised on two sheets of membrane. On the first membrane, the peptides were synthesised in six rows containing nineteen spots each and except for Phe at position 2 and Gln at position 4, the amino acid in each of the six remaining positions was changed in successive rows. Thus, in row one, a total of nineteen peptide mixtures were synthesised and in each of the mixtures, position one was defined as one of 19 amino acids and positions 2 and 4 contained Phe and Gln respectively, all the other positions contained a mixture of the 19 amino acids. In row two, another 19 group of peptide mixtures were synthesised in which position 3 was a single amino acid and as in row one positions 2 and 4 contained Phe and Gln and a mixture of 19 amino acids was used in the remaining positions. The process was repeated until all

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the single amino acids had been introduced into the six available positions. The peptides on the first membrane are represented as

O_1-Phe-X-Gln-X-X-X-X, X-Phe-O_3-Gln-X-X-X-X, X-Phe-X-Gln-O_5X-X-X, X-Phe-X-Gln-X-O_6-X-X, X-Phe-X-Gln-X-X-O_7X and X-Phe-X-Gln-X-X-X-O_8.

Thus all the permutations were achieved in 114 spots. Similarly, on the second membrane another 114 peptide mixtures were synthesised as

O_1-Ile-X-Ile-X-X-X-X; X-Ile-O_3-Ile-X-X-X-X; X-Ile-X-Ile-O_5-X-X-X-, X-Ile-X-Ile-X-O_6-X-X-; X-Ile-X-Ile-X-X-O_7X and X-Ile-X-Ile-X-X-X-O_8 respectively.

The libraries on the membrane were probed with the antibody 201/9 and although the antibody bound strongly to the peptides which contained Ile at positions 2 and 4 there was little or no effect when the residues in the other positions were changed (data not shown). This suggests that there was some interaction between these two residues and the antibody, which was independent of the amino acid sequence. In contrast, antibody binding to the peptide libraries containing Phe at position 2 and Gln at position 4 was dependent on the amino acids in the other positions (Figs 2a-f). The peptides which contained Ala or Ser in the first position bound the antibody more strongly than those with Gly or His (Fig. 2a), as judged by the densities of the spots on the film. Peptide mixtures which contained Leu in the third position (Fig. 2b) showed the strongest antibody binding, although peptides with Val or Ile in this position also bound the antibody well. The antibody bound most strongly to the peptides with Glu, Thr or Val in the fifth position (Fig. 2c), and to those with Phe or Asn in position six (Fig. 2d). The requirement for a specific amino acid at positions seven and eight was less than at the other positions. Although peptides with Gln or Asn at position seven (Fig. 2e) and Pro at position eight (Fig. 2f) bound the antibody most strongly, however, these three residues could be replaced by other neutral or hydrophobic amino acids without significantly altering the binding of the antibody. This

suggests that either at position seven and eight the amino acid requirement is less specific than at the other positions, or more likely, that the epitope does not include these residues and that the immunodominant epitope for the monoclonal antibody 201/9 is the sequence

5   Ser-Phe-Leu-Gln-Glu-Asn.

The array was re-used 20 times without any deterioration in sensitivity and specificity.

DISCUSSION

10   The membrane based peptide libraries provides a rapid and convenient technique to study the molecular interaction of a functional protein with its ligand. In addition, the residues involved in the interaction can be defined without any knowledge of the primary structure. The peptide libraries were synthesised on a piece of hydrophilic polymer

15   membrane which showed very little non-specific protein binding. Typically, only 3-4 days are required for the synthesis of the octapeptide libraries by an operator with little experience of peptide chemistry. The total number of peptides required in the first scan is 361 and in the second scan 114 and since all the peptides in each scan are present on one piece of membrane

20   only a small amount of ligand (antibody) is required for screening. Furthermore, the libraries on the membrane could be regenerated at least twenty times without a noticeable decrease in sensitivity thus enabling the same peptides to be probed by many different ligands.

A "one step" positional scanning approach to identify a

25   peptide ligand has been described. In this technique, the libraries were made up of peptide mixtures each with an amino acid defined at one position and the other positions containing a mixture of 19 amino acids. Only those peptides with the correct amino acid in each position gave the strongest recognition signal. However, in this procedure, since only one

30   residue in the sequence of the octapeptide library was defined, there would

be only one correct sequence in 19^7 (1 in 8.9×10^8) peptides. Thus from 5 mg of a peptide library a method is required to detect as little as 5.6×10^{-9} mg of the active peptide, which is beyond the limit of most detection systems. We tried unsuccessfully to identify any peptide to which

5   the monoclonal antibody 201/9 would bind using this procedure (data not shown). In the modified positional scanning approach in which two amino-acids are defined during the primary scan, the relative concentration of an individual peptide is increased so that, theoretically, 5 mg of a peptide library will yield 2×10^{-6} mg of each peptide and is well within the detection

10   limit of the chemiluminescent immunoassay. These calculations necessarily assume that the rate of coupling of each amino acid in the mixtures is the same.

Re-use of the array 20 times without any reduction in strength of signal or increase in background noise, provides a striking advantage for

15   the inventors' PVDF membranes. By comparison, cellulose membranes became unusable after as little as 3 assays.

EXAMPLE 2

Identification of Sites of Protein Protein Interaction

20   The purpose of this experiment was to investigate the sites at which β-factor XIIa interacts with the trypsin inhibitor from sweet corn. A membrane that contains a set of overlapping octapeptides based on the known sequence of β-factor XIIa was prepared. The sequence of this scanning membrane is shown in Figure 3. Various target functional

25   proteins were biotinylated and applied in solution to the scanning membrane. These functional target proteins were: monoclonal antibodies 202/6 and 202/16 and 201/9, a polycolonal antibody, and the inhibitor for β-factor XIIa. These bound to specified regions of the scanning array as shown in Figure 3 where light shading shows weak binding and dark

30   shading shows correspondingly strong binding. The peptides to which the

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various target proteins bound were identified by probing with a
streptavidin/peroxidase conjugate, and the bound conjugate was located
using a chemiluminescent assay. Preliminary work had shown that
biotinylation of the antibodies and the inhibitor had no effect on their
5 activity.

In a second experiment (results not shown) a scanning array
was prepared which contained a series of overlapping oligopeptides based
on the amino acid sequence of the inhibitor. This array was probed with
β-factor XIIa and the bound β-factor XIIa was detected using a specific
10 monoclonal antibody followed by anti-murine IgG:peroxidase conjugate
β-factor XIIa.

FIGURE LEGENDS

Fig. 1 The binding intensity of the peptide mixtures in the
15 libraries for the monoclonal antibody 201/9.

The strength of binding of the monoclonal antibody 201/9 for
the peptide mixtures with defined amino acids at positions 2 and 4 read as
a 256 grey scale is plotted against the peptide composition. X indicates
the positions containing the mixtures of amino acids.

20 Figs. 2a-f All the amino acids of the epitope identified in a one
step positional scan of the libraries.

The vertical axis represents the binding intensity on a 256
grey scale for the recognised peptide mixtures with defined amino acids.
The horizontal axis indicates the defined amino acid in the position defined
25 by the formulae:- (Fig 2a) O_1-Phe-X-Gln-X-X-X-X; (Fig 2b) X-Phe-O_3-Gln-
X-X-X-X; (Fig 2c) X-Phe-X-Gln-O_5-X-X-X; (Fig 2d) X-Phe-X-Gln-X-O_6-X-X;
(Fig 2e) X-Phe-X-Gln–X-X-O_7-X; (Fig 2f) X-Phe-X-Gln-X-X-X-O_8. Where O
is the single amino acid listed along the bottom of the figure and X denotes
a mixture of all the amino acids.

CLAIMS

5   1.   A method of studying a functional protein, which method
comprises

a)   providing an array of support-bound oligopeptides, with
different oligopeptide bound in an immunoreactive state at different
locations on the support, wherein each oligopeptide has defined amino

10   acid residues at two chosen positions,

b)   applying the functional protein in solution to the array and
identifying a first support-bound oligopeptide that binds to the functional
protein,

c)   using the information generated in b) to study the functional

15   protein.

2.   A method as claimed in claim 1, wherein each oligopeptide of
the array has defined amino acid residues at two chosen non-adjacent
positions.

3.   A method as claimed in claim 2, wherein each oligopeptide of

20   the array is support-bound through its C-terminus and the defined amino
acid residue are at the 2- and 4-positions counted from the C-terminus.

4.   A method as claimed in any one of claims 1 to 3, wherein the
amino acid residues of each oligopeptide, at all positions other than the
two chosen positions, are mixtures of all natural L-amino acids except

25   cysteine.

5.   A method as claimed in any one of claims 1 to 4, wherein
step c) is performed by providing several secondary libraries of support-
bound oligopeptides in which: each support-bound oligopeptide of each
secondary library has the same amino acid residues at the same two

30   chosen positions as the first support-bound oligopeptide; each support-

bound oligopeptide of a secondary library has a defined amino acid residue at another chosen position; and the said other chosen position is different for each secondary library; applying the functional protein in solution to each secondary library and identifying a secondary support-bound oligopeptide in each secondary library that binds to the functional proteins; and using the information generated to identify the peptide sequence of an immudominant epitope of the functional protein.

6.   A method as claimed in any one of claims 1 to 5, wherein the array comprises 361 different oligopeptides bound at 361 different locations on the support.

7.   A method as claimed in any one of claims 1 to 6, wherein the support is a polyvinylidenedifluoride membrane.

8.   An array of support-bound oligopeptides, with different oligopeptides bound in an immunoreactive state at different locations on the support, wherein each oligopeptide has defined amino acid residues at two chosen positions.

9.   An array as claimed in claim 8, wherein each oligopeptide has defined amino acid residues at two chosen non-adjacent positions.

10.   An array as claimed in claim 9, wherein each oligopeptide is support-bound through its C-terminus and the defined amino acid residues are at the 2- and 4-positions counted from the C-terminus.

11.   An array as claimed in any one of claims 8 to 10, wherein the amino acid residues of each oligopeptide, at each position other than the two chosen positions, are mixtures of all natural L-amino acids except cysteine.

12.   An array as claimed in any one of claims 8 to 11, comprising 361 different oligopeptides bound at 361 different locations on the support.

13.   An array as claimed in any one of claims 8 to 12, wherein the support is a polyvinylidenedifluoride membrane.

Fig.1.

| | | | | | | | | |
XHisXTrpXXXX								
XIleXValXXXX								
XTrpXThrXXXX								
XPheXThrXXXX								
XTyrXGlnXXXX								
XTrpXGlnXXXX								
XPheXGlnXXXX								
XTrpXProXXXX								
XTrpXAsnXXXX								
XTrpXMetXXXX								
XIleXLeuXXXX								
XIleXIleXXXX								
XTrpXHisXXXX								
XThrXHisXXXX								
XPheXHisXXXX								
XTrpXGlyXXXX								
XThrXPheXXXX								
XIleXPheXXXX								
XHisXPheXXXX								
XTrpXGluXXXX								
XTrpXAspXXXX								
XTrpXAlaXXXX								

0   32   64   96   128   160   192   224   256

BINDING INTENSITY (GREY SCALE)

Fig.2.
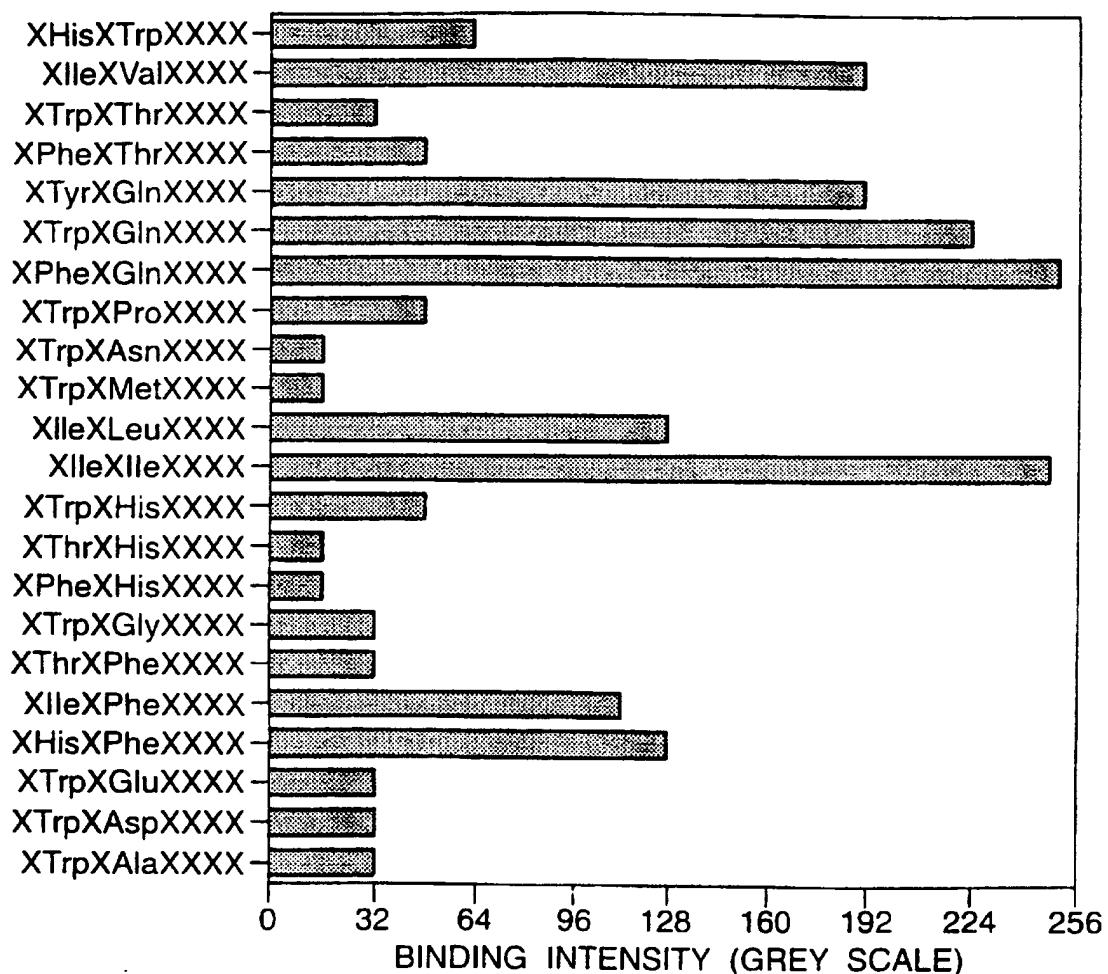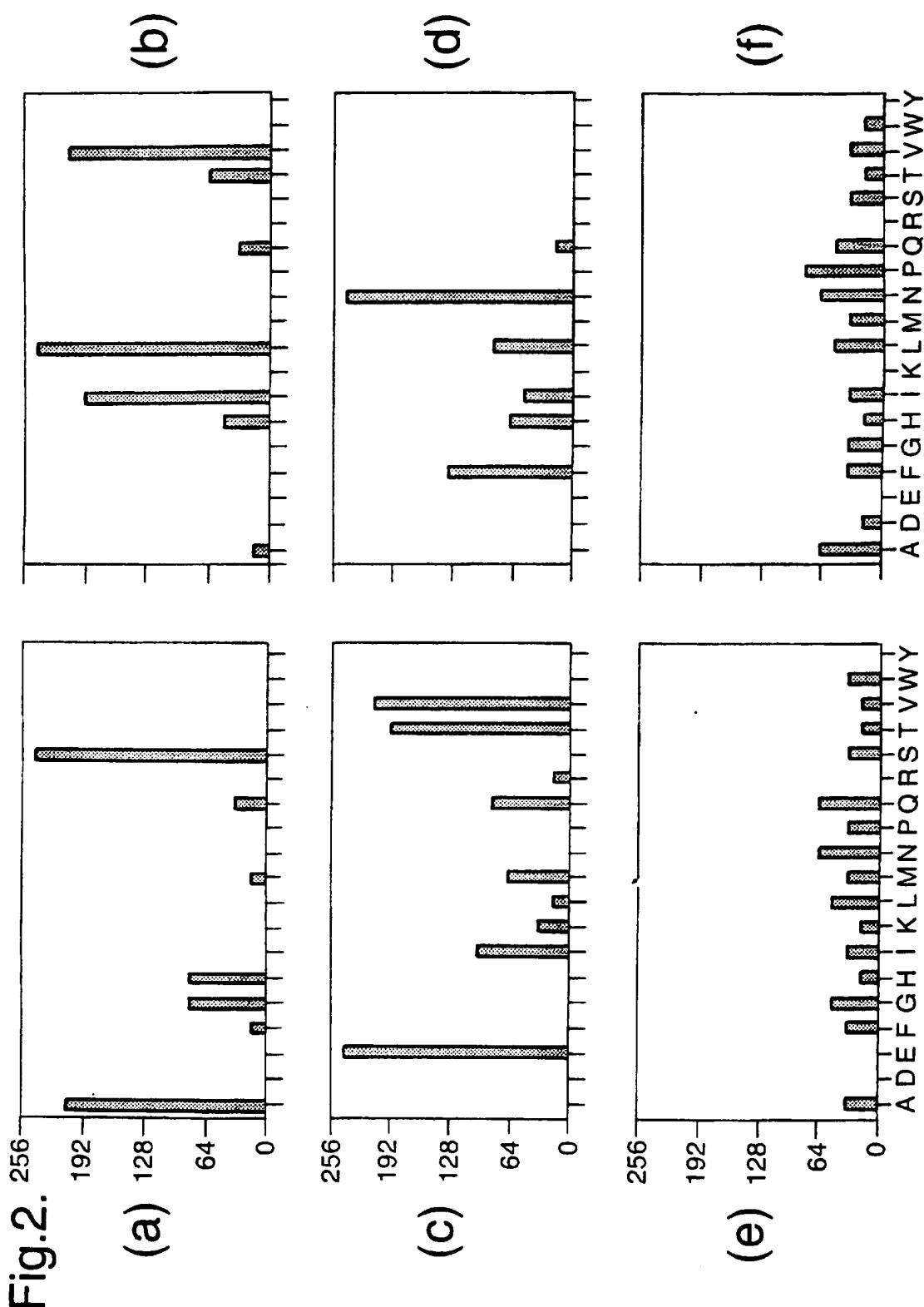
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N33/68   C07K1/04   //G01N33/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   G01N   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 86 00991 A (COMMW SERUM LAB COMMISSION) 13 February 1986	1,2,4,5, 7-9,11, 13
A	see abstract; claims & US 5 194 392 A cited in the application ---	3,10
Y	WO 94 05394 A (ARRIS PHARMACEUTICAL CORP) 17 March 1994	1,2,4,5, 7-9,11, 13
	see abstract; claims 14-17; example 5 ---	
Y	DE 43 44 425 A (SHIMADZU CORP) 30 June 1994	7,13
A	see column 2, line 17 - line 23 see column 4, line 27 - line 30 -----	1,3,10

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 August 1997	28 -08- 1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Ceder, O

Fig.3.